January 2021

Determining The Relationship Between Sars-Cov-2 Infection And Streptococcus Pneumoniae In Clinical Saliva Samples

Anne Watkins
anne.watkins6@gmail.com

Determining the relationship between SARS-CoV-2 infection and *Streptococcus pneumoniae* in clinical saliva samples

Anne E. Watkins

A thesis completed in April 2021
in partial fulfillment of the requirements for
2021 conferral of the degree of
Master of Public Health,
Yale School of Public Health

Advisor:
Anne L. Wyllie

Secondary advisor:
Nathan D. Grubaugh

Abstract

Background. *Streptococcus pneumoniae* is a commonly found upper respiratory tract colonizer that can progress to more severe disease forms such as pneumonia. Interactions with respiratory viruses other than SARS-CoV-2 have been previously identified such that *S. pneumoniae* may sometimes enhance disease or occur as a secondary or co-infection. With the recent emergence of SARS-CoV-2 there is limited data available describing this specific pathogen relationship that could play a role in the breadth and severity of the pandemic.

Methods. Inpatients and healthcare workers testing positive for SARS-CoV-2 March 2020-August 2020 were tested for *S. pneumoniae* through saliva culture enrichment and RT-qPCR as well as urine antigen detection (UAD) assays. A multinomial multivariate model was used to examine the relationship between pneumococcal presence and COVID-19 outcomes.

Results. Among 126 subjects enrolled, the median age was 62 years; 54.9% of subjects were male; 88.89% were inpatients; 23.5% had an ICU stay; and 13.5% were deceased. *S. pneumoniae* was detected in 17 subjects (13.5%) by any method, including 5 subjects (4.0%) by RT-qPCR and 12 subjects (13.6%) by UAD. Detection by UAD was highly associated with both moderate and severe COVID-19 disease while RT-qPCR detected states were not significantly predictive of such outcomes. Despite being associated with more severe outcomes, UAD positives represented 0/14 deaths within the study population.

Conclusions. Pneumococcal presence, particularly in a disease state, may be associated with more serious outcomes of SARS-CoV-2 infections. Concerns surround high levels of antibiotic usage that may diminish the ability to detect pneumococci, particularly in culture. Future studies should be performed to better characterize the relationship between these two pathogens across all levels of disease status.

Acknowledgements

First and foremost, I'd like to thank my advisors and professors who have been so supportive throughout the past two years. Dr. Anne Wyllie has been an incredible sounding board and mentor throughout my thesis and pandemic work, and I am so grateful for our weekly meetings. From one Anne to another, thank you so much for your support and being a fantastic model of women in science. Thank you as well to Dr. Nathan Grubaugh, my primary advisor and second reader, for taking the time to really sit down and provide mentorship and guidance throughout my MPH and for providing me with the opportunity to use my skills in responding to this pandemic. And to my first research advisor, Dr. Dan Weinberger, thank you for taking a chance on me early on and ensuring that I had every opportunity I could have wanted, even while on sabbatical. To Isabel Ott and Devyn Yolda-Carr, my sincerest thanks, and apologies, for all of your help with performing and prepping for some of the less appealing laboratory tasks for this project. My thanks extend to everyone in the greater Grubaugh and Weinberger labs – you all have made such an impact on my life and I hope we may be able to work together again under better, non-pandemic conditions.

To my family and friends, you are my rock. I am deeply grateful for your love, encouragement, and support throughout my MPH and beyond. Thank you, Mom and Dad, for helping me get this far and reminding me of my worth (and my life outside of COVID). Thank you for supporting a fourth grader who thought polio was really cool. My wonderful fiancé, Karl, thank you for listening to me talk endlessly about diseases and grounding me back to reality. You are such a good man, and I can't wait to spend the rest of my life with you. And last, but definitely not least, thank you Zuzu for helping me through the pandemic, you deserve an honorary MPH.

Contents

Table of Contents

Abstract 2

Acknowledgements 3

Contents 4

Tables & Figures 5

Introduction 6

Methods 9

Results & Discussion 12

References 23

Tables & Figures

Tables

Table 1. Classification of pneumococcus status. *13*
Table 2. Demographics by pneumococcal status. *17*
Table 3. Odds ratios from multinomial regression, "Moderate". *18*
Table 4. Odds ratios from multinomial regress, "Severe". *19*

Figures

Figure 1. SARS-CoV-2 N1 in comparison to both SARS-CoV-2 N2 and S. pneumoniae lytA and piaB C_T values broken down by pneumococcal status. *14*
Figure 2. Outcome Measures of COVID-19 infection categorized by pneumococcal status. *16*

Introduction

In the months following the identification of the SARS-CoV-2 virus as the cause of COVID-19 and its establishment as a pandemic, much work has been done to understand the virus, course of infection, and establish treatment and prevention policies. Most cases of COVID-19 are mild to moderate in severity while some progress to severe outcomes including pneumonia, acute respiratory distress syndrome (ARDS), and death related to a variety of risk factors.[1]

There remains limited characterization of SARS-CoV-2 interactions with both commensal bacteria and secondary infections.[2] Advantageous bacterial pneumonias, particularly *S. pneumoniae*, are often responsible for much of the severity and mortality during viral epidemics.[3-4] Prior research focuses primarily on respiratory viruses such as influenza and RSV where bacterial co-infection is relatively heterogenous in frequency and proportionality, yet *S. pneumoniae* is amongst the most common co-infecting pathogens.[5] Early data suggests that the COVID-19 pandemic may show similar low-level co-infection patterns as well as more common occurrence in ICU patients suggesting a correlation with more severe clinical outcomes.[6-7] Initial studies report any bacterial infection in approximately 6.9% of hospitalized and critically ill patients with COVID-19, as well as possible differences in bacterial composition than commonly described with other viral respiratory pathogens.[8]

For some common respiratory pathogens, such as Influenza A, a lethal synergistic relationship has been described with pneumococci.[9] Similarly, higher viral loads have been associated with more severe COVID-19 outcomes, but the interaction of bacterial co-infection on viral titers is not well characterized.[10] In conjunction with the synergistic relationship described previously, there may be an increase in relative viral titer following pneumococcal challenge; however, as a

secondary infection this may not affect the peak viral load.[11] Interestingly, an early SARS-CoV-2 case study from Japan noted co-infection with *S. pneumoniae* that had the appearance of lobe-level pathogen mutual exclusion such that *S. pneumoniae* was primarily found in the lower lungs and SARS-CoV-2 in the upper lobes.[12]

To best address case outcomes and disease severity a more complete understanding of these microbial interactions is necessary. Upper respiratory tract carriage of *S. pneumoniae* is typically asymptomatic and can be a part of the normal flora; however, this colonization state can lead to disease states such as pneumonia or meningitis.[13] Pneumococcal carriage and disease rates in the community are highest amongst children[14] and the elderly,[15] respectively, both groups of particular concern throughout the pandemic. Should *S. pneumoniae* carriage or progression to disease be associated with more severe COVID-19 outcomes these individuals may face an even greater risk. Furthermore, development of pneumococcal disease or even detection of carriage could lead to greater levels of broad-spectrum antibiotic use in patients that could exacerbate problems of antimicrobial resistance.[16-17] Antimicrobial stewardship remains a complex and important factor in mitigating current and future populational infectious threats.

Not only is there the potential for invasive, secondary infections from pneumococcus of concern in relation to SARS-CoV-2 infection, there could additionally be a relationship with common serotypes seen in carriage to enhanced disease severity.[18-19] Contrarily, high carriage density has also been shown to be protective against respiratory syncytial virus (RSV) disease severity in infants related to the immune response.[20] As of now, the within-host biological interactions of *S. pneumoniae* and SARS-CoV-2 that could influence disease progression and pathology have yet to be well characterized. Pneumococcal vaccines have been shown to have some indirect effect

on viral infections previously and could be highly influential should there be a relationship between SARS-CoV-2 and vaccine-type serotypes.[21-22] Furthermore, this may be of particular importance with age and comorbidity-related morbidity and mortality risk in both pneumococcal and SARS-CoV-2 infections.[23-24]

With the retrospective cohort of SARS-CoV-2 positive inpatients and healthcare workers available through the Yale IMPACT (IMplementing Public health Action against coronavirus CT) biorepository, we aim to identify the prevalence of pneumococcus within this population and explore its association with SARS-CoV-2. Based on pneumococcal interactions with other viral respiratory diseases there is reason for concern that pneumococcal presence could lead to more severe outcomes that must be explored within the specific context of SARS-CoV-2 infection and its pandemic spread. The role of these potential relationships is important in considering public health response measures that could be used to mitigate both pneumococcal disease and COVID-19.

Methods

Study design. During March-August of 2020 de-identified saliva and urine samples were collected from SARS-CoV-2 positive inpatients and healthcare workers on COVID-19 units at Yale-New Haven Hospital as part of the IMPACT biorepository.[25] Signed-informed consent was obtained from all study participants following Yale University HIC-approved protocol #2000027690. Demographic information was collected as part of biorepository efforts. Amongst the inpatients and healthcare workers identified and enrolled in the biorepository, 126 individuals who tested positive for SARS-CoV-2 had saliva samples remaining for testing in this study.

Sample collection. Inpatient saliva self-collection was attempted every three days using the methods previously described.[26] Likewise, healthcare workers were asked to collect saliva and nasopharyngeal swab samples as often as every three days for up to 84 days or testing positive for SARS-CoV-2. Samples were stored at room temperature and transported to the Yale School of Public Health within 5 hours of sample collection and tested for SARS-CoV-2 within 12 hours of sample collection. Urine collection was also attempted every three days from enrolled participants and stored at -80˚C until further processing.

SARS-CoV-2 RNA detection in saliva. Nucleic acid was extracted from 300 µl of saliva using the MagMAX Viral/Pathogen Nucleic Acid Isolation kit (ThermoFisher Scientific) on the KingFisher DNA extraction robot (ThermoFisher Scientific) following a modified protocol.[26] Samples were classified as positive for SARS-CoV-2 when both N1 and N2 targets were detected with <40 C_T by RT-qPCR. Where possible, 100 ul of the remaining sample volume was supplemented with 20 µl brain heart infusion (BHI) supplemented with 50% glycerol and stored at -80˚C for further analysis.[25]

Culture enrichment and detection of pneumococcal carriage using the molecular method. For all COVID-19 inpatients and for healthcare workers with at least one SARS-CoV-2 positive test, by any sample type, remaining saliva samples were tested for pneumococcal carriage. Both raw and BHI/10% glycerol supplemented saliva samples were first culture enriched with 100 µl on gentamicin (10%) supplemented blood agar plates. [27] Cultures were incubated overnight after which all growth was harvested into 2100 µl of BHI supplemented with 10% glycerol and stored at -80˚C. Pneumococcal detection was performed by DNA extraction of 200 µl of each sample using the MagMAX Ultra Viral/Pathogen Nucleic Acid Isolation kit (ThermoFisher Scientific) on the KingFisher DNA extraction robot (ThermoFisher Scientific) following manufacturer's protocol. Samples were classified as positive when both *piaB*[28-29] and *lytA*[30] targets were <40 C_T by RT-qPCR.

Urine antigen detection (UAD). Urine samples were thawed at room temperature and aliquoted into PIPES buffer. Aliquots were re-frozen at -80˚C in preparation for batch shipping on dry ice to the reference laboratory of Pfizer Vaccine Research (Pearl River, NY) for testing[31]. Upon receipt, samples were stored at -80˚C until batched urine antigen testing could be performed using the serotype-specific UAD and/or BinaxNOW tests according to manufacturer's protocol.

Statistical analysis. Differences in demographic data between pneumococcal status groups were tested using analysis of variance F-test (continuous variable) or χ^2 test (categorical variable). Multivariate and multinomial logistic regression analyses were used to evaluate the association SARS-CoV-2 infection outcome levels with pneumococcal status. The outcome variable was trichotomous for whether an individual was mild/asymptomatic, moderate, and severe disease as defined by clinical scoring using a custom disease severity scale.[37] The model was pared down

according to AIC scores and odds ratio confidence intervals between versions. Covariates included in the final version were pneumococcal status (negative, positive by RT-qPCR, indeterminant – *lytA* by RT-qPCR, positive or indeterminant by UAD), age, sex, BMI, and race. Model coefficients and confidence intervals were exponentiated to transform into odds. Estimates were considered statistically significant at $p<0.05$. All statistical analyses were performed in RStudio v1.2.1335[35], using R v3.6.1.[36]

Results & Discussion

Pneumococcal Detection

A total of 235 saliva samples from 126 SARS-CoV-2 positive individuals, comprised of 14 healthcare workers (HCW) and 112 inpatients (INP), were culture enriched and tested by RT-qPCR for *S. pneumoniae*. Of these, eight samples, representing five individuals, tested positive for both bacterial targets suggesting a pneumococcal carriage rate of 4.0% amongst those with SARS-CoV-2. A further 11 individuals tested positive for *lytA* alone suggesting possible streptococcal carriage of 12.7%. Additionally, 159 inpatients were tested for *S. pneumoniae* by Urine Antigen Detection (UAD), 88 of whom were also tested by saliva. Of these, 15 individuals (9.4%) tested positive for at least one pneumococcal polysaccharide antigen, 12 of whom were also tested by saliva (13.6%). Furthermore, three individuals were indeterminant by UAD with one simultaneously tested by saliva.

The outcomes of both culture enrichment/RT-qPCR and UAD tests were used to classify pneumococcus status (Table 1) in order to further explore the relationships between these samples. Amongst the different pneumococcal statuses there does not appear to be any association with SARS-CoV-2 N1 or N2 cycle threshold (C_T) values in saliva (Figure 1). There is no apparent correlation between N1 and *lytA* (R=-0.363) or *piaB* (0.194) C_T values, when both detected, suggesting that viral load may not affect pneumococcal abundance. Likewise, all such true pneumococci were detectable in the first saliva samples collected indicating pre-existing carriage with latest detection occurring in early May. UAD positives, however, occurred throughout the course of infection suggesting possible acquisition during the course of stay with detection spread throughout most of the study period. Incidence of *S. pneumoniae* by UAD

(13.6%) is highly similar to levels of detection seen amongst older adults with general community-acquired pneumonias seen throughout the U.S. previously[35] suggesting that secondary infections in COVID-19 patients may be on par with what has previously been described.

Table 1. Classification of pneumococcus status.

Pneumococcus Status	Definition
0	Not detected by any measure
1	RT-qPCR: *lytA* C_T <40; *piaB* C_T <40
2	RT-qPCR: *lytA* C_T <40; *piaB* C_T >40 or not detected
3	UAD positive or indeterminant

Notably, there was no concordance for pneumococcal detection between positives and indeterminants within individuals tested by both methods. This lack of concordance could reflect detection of two separate types of pneumococcal events with differing implications for overall outcomes and may be explained in part by the testing methods themselves. UAD is designed to particularly detect pneumococcal pneumonia rather than carriage,[31] establishing this classification status as a secondary infection compared to the culture enrichment based statuses that suggest carriage. Additionally, culture enrichment steps limit detection to living bacteria in saliva[36] and does not account for disruption of *S. pneumoniae* colonization, such as by antibiotic usage, leading to the presence of killed bacteria that could be detected by DNA extraction alone. Evidence of mutually exclusive lung pathology within co-infection[12] states with *S. pneumoniae* primarily residing in the lower lobes of the lung supports the idea that saliva-based culture enrichment may not be able to as readily identify pneumococcal pneumonias due to lack of bacterial colonization in the upper respiratory tract. Future studies with both greater sample

collection volumes, preferably 1.5mL or greater, and greater participant enrollment could combine all of these techniques to better identify and tease apart these coinfections.

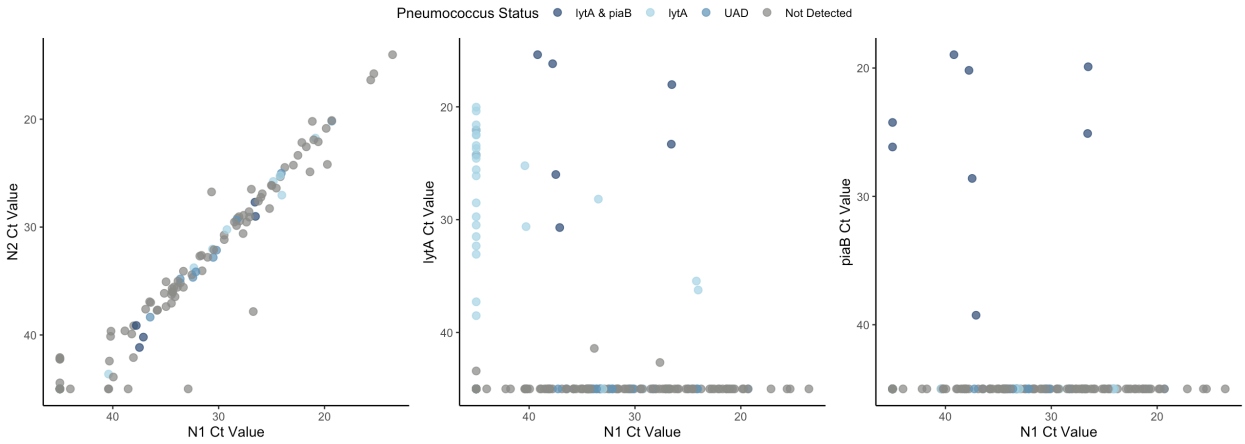

Figure 1. SARS-CoV-2 N1 in comparison to both SARS-CoV-2 N2 and *S. pneumoniae lytA* and *piaB* C_T values broken down by pneumococcal status. A positive trend can be seen between N1 and N2 C_T values as expected with consistent within-host viral load. Pneumococcal gene targets can be seen throughout a spectrum of SARS-CoV-2 detection levels and appear largely independent from viral load.

Overall, pneumococcal carriage estimates (4.0%) within this study population are low relative to rates reported throughout the literature for non-elderly and elderly adults alike, but particularly in older adults with influenza-like illnesses which this study population most closely reflects.[27,37] It is likely that a number of factors are contributing to this discrepancy apart from SARS-CoV-2 infection. With the majority of participants enrolled due to COVID-19 inpatient status, it is possible that many may have been provided antibiotics upon admission or during their stay. Additionally, pandemic response measures such as social distancing and masking minimized contact between individuals that may have driven transmission and colonization, particularly between grandchildren and their grandparents.[38] Furthermore, related to the timing of the pandemic itself, samples were collected March-August 2020 while most studies estimating pneumococcal carriage are typically performed during the peak respiratory pathogen seasons of fall and winter which may show greater levels of carriage.

Demographics

Key demographical differences exist amongst the pneumococcal status groups (Table 2). Amongst the four primary bacterial classifications detailed, significant differences were identified in the categories of family size and race/ethnicity (α=0.05) by ANOVA or χ^2. The strep/pneumococcal carriage states participants were, on average, younger and had more family members living in their home possibly indicating a parental role or at least children in the home. Individuals spending more time around children may help explain their carrier state apart from SARS-CoV-2 infection.[37] Conversely, in these factors UAD positive individuals look much more like those without any form of pneumococcal detection supporting UAD as a possible secondary infection acquired in the hospital or community rather than a form of carriage. Additionally, UAD classification shows the most distinct racial grouping compared to all of the other detection statuses. This subset is predominately black while the others are mostly white, although the pneumococcal carriage (*lytA* and *piaB* positive) group was evenly distributed along racial lines. Based on summary demographics alone it is difficult to determine the relationship between pneumococcal pneumonia, severity of COVID-19, and race, but this raises important questions on the disproportionate burden of disease and health equity. Furthermore, the small sample size of study participants and individual pneumococcal statuses increases challenges in identifying whether these differences are clinically meaningful or a product of sampling biases.

Pneumococcal status as a predictor of SARS-CoV-2 outcomes

The "case status" variable was selected as a primary outcome measure due to completeness of data and more consistent classification of SARS-CoV-2 outcomes than deaths or admission to ICU. This measure appears to have a relationship with pneumococcus status (Figure 2), UAD positives being of particular note in severe classification compared to other bacterial states.

Interestingly, none of those who were UAD positive died despite being primarily comprised of severe cases while 11/31 (35.5%) of non-pneumococcal severe cases are known to be deceased. At this time it is difficult to say with certainty why such a difference in case fatality may be occurring. One possible explanation may relate to available courses of treatment and classification systems such that any form of pneumonia is more readily classified as severe disease while a secondary, pneumococcal pneumonia may be more treatable than a primary COVID-19 pneumonia. While some patients will progress to UAD positive pneumococcal pneumonia state, many will already be on or can have near-immediate antibiotic starts.

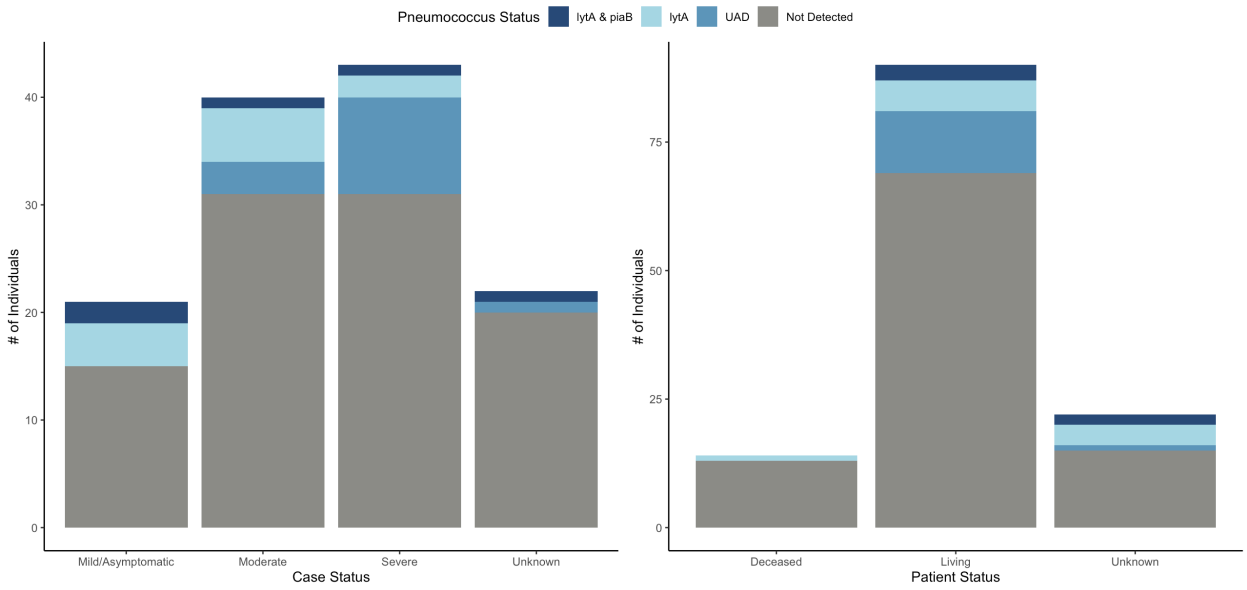

Figure 2. Outcome Measures of COVID-19 infection categorized by pneumococcal status. UAD positive individuals are disproportionally categorized as severe disease, particularly in comparison to the other pneumococcal statuses. Despite this, deceased individuals primarily have no detection of *S. pneumoniae* and no UAD positives died while hospitalized for COVID-19.

In order to broadly examine the role that pneumococcal status may play in clinical outcomes, broken down as asymptomatic/mild, moderate, and severe disease, a multivariate multinomial model was developed. Additional covariates were included in order to adjust for other factors that may influence both COVID-19 outcomes and pneumococcal status. After adjustment – increasing age, male sex, and UAD positive status suggest an increased risk of both moderate

Table 2. Demographics by pneumococcal status.

Characteristic	No Pneumococcal Detection (n = 97)	*lytA* & *piaB* Positive (n = 5)	*lytA* Positive (n = 11)	UAD Positive or Indeterminant (n=13)	Any Detection (n = 29)	p†
Age (years), mean ± SD	58.7 ± 18.3	54.8 ± 20.4	46.1 ± 15.3	59.2± 21.1	53.5 ± 19.3	0.190
BMI, mean ± SD	31.9 ± 7.9	31.0 ± 11.7	29.0 ± 5.8	29.0 ± 7.3	29.3 ± 7.2	0.500
Family Size, mean ± SD	1.1 ± 1.5	3.8 ± 4.5	2.1 ± 1.6	0.7 ± 1.0	1.7 ± 2.3	0.005
Sex						
Female	42 (45.2)	3 (60.0)	3 (27.3)	7 (53.8)	13 (44.8)	0.520
Male	51 (54.8)	2 (40.0)	8 (72.7)	6 (46.3)	16 (55.2)	
Patient Type, n (%)						0.172
HCW	10 (10.3)	1 (20.0)	3 (27.3)	0 (0.0)	4 (13.8)	
INP	87 (89.7)	4 (80.0)	8 (72.7)	13(100.0)	25 (86.2)	
Admitted to ICU, n (%)						0.263
No	58 (76.3)	3 (75.0)	8 (100.0)	6 (60.0)	17 (77.3)	
Yes	18 (23.7)	1 (25.0)	0 (0.0)	4 (40.0)	5 (22.7)	
Patient Status, n (%)						0.433
Living	69 (84.1)	3 (100.0)	6 (85.7)	12 (100.0)	21 (95.5)	
Deceased	13 (15.9)	0 (0.0)	1 (14.3)	0 (0.0)	1 (4.5)	
Case Status, n (%)						0.072
Asymptomatic/Mild	15 (19.5)	2 (50.0)	4 (36.4)	0 (0.0)	6 (22.2)	
Moderate	31 (40.3)	1 (25.0)	5 (45.5)	3 (25.0)	9 (33.3)	
Severe	31 (40.3)	1 (25.0)	2 (18.2)	9 (75.0)	12 (44.4)	
Race/Ethnicity, n (%)						0.003
Non-Hispanic white	38 (50.7)	1 (33.3)	7 (70.0)	1 (10.0)	9 (39.1)	
Non-Hispanic black	23 (30.7)	1 (33.3)	0 (0.0)	9 (90.0)	10 (43.5)	
Hispanic	14 (18.7)	1 (33.3)	3 (30.0)	0 (0.0)	4 (17.4)	

* Numbers may not sum to totals due to missing data, and column percentages may not sum to 100% due to rounding.

† P-value for analysis of variance F-test (continuous variable) or χ^2 test (categorical variable) between individual pneumococcal levels, not including "Any Detection".

and severe disease with odds ratios of 1.13/1.16, 11.35/30.68, and 2926.91/8252.83 (moderate/severe), respectively (Tables 3&4). Carriage-based pneumococcal statuses were not predictive of clinical severity level in this model. To this extent, further questions can be raised as to whether a UAD positive status is predictive of case status or if the exact opposite is true. More severe cases of COVID-19 may be more likely to develop a secondary pneumococcal pneumonia due to existing disease pathology. The particularities of this relationship will require more in-depth future study in both epidemiological and molecular mechanisms.

With such sparse data overall, it is difficult to confidently describe the effects of pneumococcal status on COVID-19 outcomes. A variety of issues arise with running the model in its current form, but of primary concern is the data stratification of already small sample sizes. For example, the odds ratios of UAD positive status are much higher than one would expect in part due to no individuals in the mild /asymptomatic reference outcome. Ideally, more individuals of all pneumococcal classifications would be included in this study to more completely assess the role of *S. pneumoniae* in the SARS-CoV-2 pandemic.

Table 3. Odds ratios from multinomial regression, "Moderate".

Characteristic	Adjusted OR (95% CI)	p
Pneumococcus Status		
No Detection	1.00	---
lytA & piaB	0.98 (0.00, 287.72)	0.995
lytA	0.94 (0.05, 16.71)	0.967
UAD Positive	2926.91 (1279.16, 6697.22)	<0.001
Age	1.13 (1.05, 1.21)	<0.001
Sex		
Female	1.00	---
Male	11.35 (1.46, 88.38)	0.020
BMI	1.04 (0.89, 1.22)	0.608
Race		
Non-Hispanic white	1.00	---
Non-Hispanic black	3.47 (0.14, 88.51)	0.452
Hispanic	7.16 (0.61, 83.47)	0.116

Table 4. Odds ratios from multinomial regress, "Severe".

Characteristic	Adjusted OR (95% CI)	p
Pneumococcus Status		
No Detection	1.00	---
lytA & *piaB*	0.47 (0.00, 342.06)	0.824
lytA	0.17 (0.00, 6.34)	0.340
UAD Positive	8252.83 (3607.77, 18878.46)	<0.001
Age	1.16 (1.07, 1.25)	<0.001
Sex		
Female	1.00	---
Male	30.68 (3.30, 285.04)	0.003
BMI	1.12 (0.95, 1.32)	0.181
Race		
Non-Hispanic white	1.00	---
Non-Hispanic black	7.72 (0.29, 208.24)	0.224
Hispanic	15.15 (1.03, 223.89)	0.048

Serotypes and pneumococcal vaccines

While serotyping could not be performed for the culture enriched samples due to time and resource constraints, 8/12 of the UAD positives samples, excluding the one indeterminant, were run on the serotype-specific UAD test. Of these eight samples, three were identified as serotypes covered in the pneumococcal conjugate vaccine (PCV13) and all eight are covered in the pneumococcal polysaccharide vaccine, (PPSV23). Future explorations should consider pneumococcal vaccination status when aiming to look at serotype-specific patterns and effects. It is unlikely that most individuals in this study would have received either vaccine due to timing of vaccine introductions and age-based recommendations.[39] Approximately 33% of individuals included in the study are estimated to have qualified for vaccination either in childhood or older adulthood, with a slightly higher proportion amongst those who were UAD positive.

Antibiotic resistance and stewardship

Of the utmost importance in collecting accurate data on pneumococcal status is antibiotic usage, particularly for inpatients. These data were not available without extensive chart review through the biorepository; however, studies suggest that general use in inpatients may be upwards of 70%.[16] There is anecdotal evidence of this within the study population (S. Farhadian, personal communication, February 1, 2021) and in a number of samples that showed little to no bacterial growth when cultured despite adequate sample volume and incubation time. Culture enrichment should produce a solid lawn of bacterial growth due to the polymicrobial nature of saliva. Minimal growth by these methods indicates a large disruption of the bacterial flora, likely due to antibiotic use. Should such a high level of antibiotic usage be the case amongst COVID-19 patients enrolled then our estimates of pneumococcal prevalence are likely minimized and do not allow for adequate stratification of individuals that introduces biases to the analysis of implications for disease severity. These data will be necessary to understanding the relationship between these two pathogens in disease progression and pathology. Most of the literature available discusses *S. pneumoniae* with other respiratory viruses which present good initial comparisons but fail to address the intricacies of SARS-CoV-2 specifically. Determining prevalence of co-infection, of any type, can justify further studies into biological mechanisms and relationships, so it is vital to tease apart whether this is low because of preemptive antibiotic usage rather than pathogen-pathogen interactions within an individual.

Even in the midst of a pandemic that continues to rage on, public health and medical officials must remain vigilant in maintaining antibiotic stewardship. Heavy antibiotic use in COVID-19 wards may be warranted if secondary bacterial infections, pneumococcal or otherwise, are common. However, if rates are lower or secondary infection can be identified easily and early on

then proactive prescribing should be discontinued, particularly in lower risk groups. With rising antibiotic resistance across many bacteria and without indication of necessity in patients, this outdated practice seemingly makes broader public health problems worse without actually improving patient outcomes.

Future Directions

One of the major limitations of this study is small sample size and incomplete demographic and medical data. With the extensiveness of the pandemic, future studies could be expanded to include more individuals of varying disease severities and demographic backgrounds with more complete sample and data collection. This could be done both retrospectively through chart reviews and analysis of previously stored samples or prospectively with new cases and data collection. Future studies should strive to have the most complete data possible on demographics, medical history, and treatments used for all enrolled individuals. Increasing longitudinal saliva and urine collection during both inpatient and outpatient stay to monitor both existing and newly acquired carriage as well as secondary infection throughout the duration of disease could help inform important questions on the roles of these different types of pneumococcal states. The methods and analyses detailed throughout this paper could function as a basic starting point for this sort of ongoing development. Furthermore, a comprehensive longitudinal surveillance study for both the general community and inpatients would best inform the dynamics and interconnectedness of SARS-CoV-2 and *S. pneumoniae* as well as other respiratory pathogens. A large research institution with corresponding healthcare system may be able to work together to tack this onto COVID-19 surveillance programs to sustainable serve the community and public health in both real time and research that will be of future benefit.

Conclusion

In conclusion, a low prevalence of pneumococcal carriage was identified amongst SARS-CoV-2 positive individuals when molecular methods where used. A higher prevalence of pneumococcal pneumonia was seen within the same population and without overlap. Furthermore, this pneumococcal disease state was associated with more severe cases and outcomes of COVID-19, while carriage did not increase these odds. Detection of pneumococci may be biased by inpatient treatments, seasonal timing, and public health measures that could mask true pathological relationships. High levels of antibiotic use may be a contributing factor to this masking which could have implications for antimicrobial stewardship and the growing problem of drug resistance. Future studies should aim to more deeply and longitudinally explore these dynamics in order to better define the relationship between these pathogens and implement both clinical and public health responses.

References

1. Gupta, S., Hayek, S. S., Wang, W., Chan, L., Mathews, K. S., Melamed, M. L., ... & Reiser, J. (2020). Factors associated with death in critically ill patients with coronavirus disease 2019 in the US. *JAMA internal medicine*.
2. Rawson, T. M., Moore, L., Zhu, N., Ranganathan, N., Skolimowska, K., Gilchrist, M., Satta, G., Cooke, G., & Holmes, A. (2020). Bacterial and fungal co-infection in individuals with coronavirus: A rapid review to support COVID-19 antimicrobial prescribing. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, ciaa530. Advance online publication. https://doi.org/10.1093/cid/ciaa530
3. Morens, D. M., Taubenberger, J. K., & Fauci, A. S. (2008). Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *The Journal of infectious diseases*, *198*(7), 962–970. https://doi.org/10.1086/591708
4. McCullers JA. The co-pathogenesis of influenza viruses with bacteria in the lung. Nat Rev Microbiol. 2014 Apr;12(4):252-62. doi: 10.1038/nrmicro3231. Epub 2014 Mar 3. PMID: 24590244.
5. Klein, E. Y., Monteforte, B., Gupta, A., Jiang, W., May, L., Hsieh, Y. H., & Dugas, A. (2016). The frequency of influenza and bacterial coinfection: a systematic review and meta-analysis. *Influenza and other respiratory viruses*, *10*(5), 394-403.

6. Lehmann, C. J., Pho, M. T., Pitrak, D., Ridgway, J. P., & Pettit, N. N. (2020). Community acquired co-infection in COVID-19: a retrospective observational experience. *Clinical Infectious Diseases*.
7. Zhu, X., Ge, Y., Wu, T., Zhao, K., Chen, Y., Wu, B., Zhu, F., Zhu, B., & Cui, L. (2020). Co-infection with respiratory pathogens among COVID-2019 cases. *Virus research*, *285*, 198005. https://doi.org/10.1016/j.virusres.2020.198005
8. Langford, B. J., So, M., Raybardhan, S., Leung, V., Westwood, D., MacFadden, D. R., ... & Daneman, N. (2020). Bacterial co-infection and secondary infection in patients with COVID-19: a living rapid review and meta-analysis. *Clinical Microbiology and Infection*.
9. Franz, A., Adams, O., Willems, R., Bonzel, L., Neuhausen, N., Schweizer-Krantz, S., ... & Tenenbaum, T. (2010). Correlation of viral load of respiratory pathogens and co-infections with disease severity in children hospitalized for lower respiratory tract infection. *Journal of clinical virology*, *48*(4), 239-245.
10. Liu, Y., Yan, L. M., Wan, L., Xiang, T. X., Le, A., Liu, J. M., ... & Zhang, W. (2020). Viral dynamics in mild and severe cases of COVID-19. *The Lancet Infectious Diseases*.
11. Smith, A. M., Adler, F. R., Ribeiro, R. M., Gutenkunst, R. N., McAuley, J. L., McCullers, J. A., & Perelson, A. S. (2013). Kinetics of coinfection with influenza A virus and Streptococcus pneumoniae. *PLoS Pathog*, *9*(3), e1003238.
12. Tsukamoto, T., Nakajima, N., Sakurai, A., Nakajima, M., Sakurai, E., Sato, Y., ... & Suzuki, T. Early Release-Lung Pathology of Mutually Exclusive Co-infection with SARS-CoV-2 and Streptococcus pneumoniae.

13. Bogaert, D., de Groot, R., & Hermans, P. W. M. (2004). Streptococcus pneumoniae colonisation: the key to pneumococcal disease. *The Lancet infectious diseases*, *4*(3), 144-154.

14. Bosch, A. A., van Houten, M. A., Bruin, J. P., Wijmenga-Monsuur, A. J., Trzciński, K., Bogaert, D., ... & Sanders, E. A. (2016). Nasopharyngeal carriage of Streptococcus pneumoniae and other bacteria in the 7th year after implementation of the pneumococcal conjugate vaccine in the Netherlands. *Vaccine*, *34*(4), 531-539.

15. Janssens, J. P., & Krause, K. H. (2004). Pneumonia in the very old. *The Lancet infectious diseases*, *4*(2), 112-124.

16. Langford, B. J., So, M., Raybardhan, S., Leung, V., Westwood, D., MacFadden, D. R., ... & Daneman, N. (2020). Bacterial co-infection and secondary infection in patients with COVID-19: a living rapid review and meta-analysis. *Clinical Microbiology and Infection*.

17. Youngs, J., Wyncoll, D., Hopkins, P., Arnold, A., Ball, J., & Bicanic, T. (2020). Improving antibiotic stewardship in COVID-19: bacterial co-infection is less common than with influenza. *The Journal of Infection*.

18. Greenberg D, Givon-Lavi N, Faingelernt Y, Ben-Shimol S, Avni YS, Bar-Ziv J, Dagan R. Nasopharyngeal Pneumococcal Carriage During Childhood Community-Acquired Alveolar Pneumonia: Relationship Between Specific Serotypes and Coinfecting Viruses. J Infect Dis. 2017 Apr 1;215(7):1111-1116. doi: 10.1093/infdis/jiw613. PMID: 28011920.

19. Weinberger, D. M., Harboe, Z. B., Viboud, C., Krause, T. G., Miller, M., Mølbak, K., & Konradsen, H. B. (2013). Serotype-specific effect of influenza on adult invasive pneumococcal pneumonia. *The Journal of infectious diseases*, *208*(8), 1274-1280.

20. Vissers, M., Ahout, I. M., van den Kieboom, C. H., Groh, L., Cremers, A. J., de Groot, R., ... & Ferwerda, G. (2016). High pneumococcal density correlates with more mucosal inflammation and reduced respiratory syncytial virus disease severity in infants. *BMC infectious diseases*, *16*(1), 1-8.

21. Simonsen, L., Taylor, R. J., Young-Xu, Y., Haber, M., May, L., & Klugman, K. P. (2011). Impact of pneumococcal conjugate vaccination of infants on pneumonia and influenza hospitalization and mortality in all age groups in the United States. *mBio*, *2*(1), e00309–e310. https://doi.org/10.1128/mBio.00309-10

22. Caldwell, R., Roberts, C. S., An, Z., Chen, C. I., & Wang, B. (2015). The health and economic impact of vaccination with 7-valent pneumococcal vaccine (PCV7) during an annual influenza epidemic and influenza pandemic in China. *BMC infectious diseases*, *15*, 284. https://doi.org/10.1186/s12879-015-1021-x

23. Pelton, S. I., Shea, K. M., Weycker, D., Farkouh, R. A., Strutton, D. R., & Edelsberg, J. (2015). Rethinking risk for pneumococcal disease in adults: the role of risk stacking. *Open forum infectious diseases*, *2*(1), ofv020. https://doi.org/10.1093/ofid/ofv020

24. Van Buynder, P., & Booy, R. (2018). Pneumococcal vaccination in older persons: where are we today?. *Pneumonia*, *10*(1), 1.

25. Wyllie, A. L., Fournier, J., Casanovas-Massana, A., Campbell, M., Tokuyama, M., Vijayakumar, P., ... & Ko, A. I. (2020). Saliva or nasopharyngeal swab specimens for detection of SARS-CoV-2. *New England Journal of Medicine*, *383*(13), 1283-1286.

26. Ott, I. M., Vogels, C., Grubaugh, N., & Wyllie, A. L. (2020). Saliva collection and RNA extraction for SARS-CoV-2 detection. *protocols. io*.

27. Krone, C. L., Wyllie, A. L., van Beek, J., Rots, N. Y., Oja, A. E., Chu, M. L. J., ... & Trzciński, K. (2015). Carriage of Streptococcus pneumoniae in aged adults with influenza-like-illness. *PloS one*, *10*(3), e0119875.

28. Wyllie, A. L., Wijmenga-Monsuur, A. J., Van Houten, M. A., Bosch, A. A., Groot, J. A., van Engelsdorp Gastelaars, J., ... & Trzciński, K. (2016). Molecular surveillance of nasopharyngeal carriage of Streptococcus pneumoniae in children vaccinated with conjugated polysaccharide pneumococcal vaccines. *Scientific reports*, *6*(1), 1-9.

29. Trzciński, K., Bogaert, D., Wyllie, A., Chu, M. L. J., van der Ende, A., Bruin, J. P., ... & Sanders, E. A. (2013). Superiority of trans-oral over trans-nasal sampling in detecting Streptococcus pneumoniae colonization in adults. *PloS one*, *8*(3), e60520.

30. Maria da Gloria, S. C., Tondella, M. L., McCaustland, K., Weidlich, L., McGee, L., Mayer, L. W., ... & Sampson, J. S. (2007). Evaluation and improvement of real-time PCR assays targeting lytA, ply, and psaA genes for detection of pneumococcal DNA. *Journal of clinical microbiology*, *45*(8), 2460-2466.

31. Huijts, S. M., Pride, M. W., Vos, J. M., Jansen, K. U., Webber, C., Gruber, W., ... & Bonten, M. J. (2013). Diagnostic accuracy of a serotype-specific antigen test in community-acquired pneumonia. *European Respiratory Journal*, *42*(5), 1283-1290.

32. Lucas, C., Wong, P., Klein, J., Castro, T. B., Silva, J., Sundaram, M., ... & Iwasaki, A. (2020). Longitudinal analyses reveal immunological misfiring in severe COVID-19. *Nature*, *584*(7821), 463-469.

33. RStudio Team (2018). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.

34. R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

35. Sherwin, R. L., Gray, S., Alexander, R., McGovern, P. C., Graepel, J., Pride, M. W., ... & File Jr, T. M. (2013). Distribution of 13-valent pneumococcal conjugate vaccine Streptococcus pneumoniae serotypes in US adults aged≥ 50 years with community-acquired pneumonia. *The Journal of infectious diseases*, *208*(11), 1813-1820.

36. Wyllie, A. L., Chu, M. L. J., Schellens, M. H., van Engelsdorp Gastelaars, J., Jansen, M. D., van der Ende, A., ... & Trzciński, K. (2014). Streptococcus pneumoniae in saliva of Dutch primary school children. *PloS one*, *9*(7), e102045.

37. Wyllie, A. L., Rümke, L. W., Arp, K., Bosch, A. A., Bruin, J. P., Rots, N. Y., ... & Trzciński, K. (2016). Molecular surveillance on Streptococcus pneumoniae carriage in non-elderly adults; little evidence for pneumococcal circulation independent from the reservoir in children. *Scientific reports*, *6*(1), 1-9.

38. Ansaldi, F., De Florentiis, D., Canepa, P., Ceravolo, A., Rappazzo, E., Iudici, R., ... & Durando, P. (2013). Carriage of Streptoccoccus pneumoniae in healthy adults aged 60 years or over in a population with very high and long-lasting pneumococcal conjugate vaccine coverage in children: Rationale and perspectives for PCV13 implementation. *Human vaccines & immunotherapeutics*, *9*(3), 614-620.

39. Centers for Disease Control and Prevention. (2020, September 1). *Pneumococcal disease surveillance reporting and trends*. https://www.cdc.gov/pneumococcal/surveillance.html.